Identification and characterization of genes regulating sensory neurogenesis and differentiation
by Branden Ray Nelson

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry
Montana State University

Abstract:
Vertebrate neurogenesis occurs through the restricted expression of subsets of genes to discrete cell types that organize into the central and peripheral nervous systems during specific developmental periods. A major goal of developmental biology is to understand the molecular and cellular mechanisms underlying this complex process. An experimentally rich system in which to investigate this question is the dorsal root ganglion (DRG), which derives from a population of migrating neural crest cells that aggregate laterally to the neural tube, and contain the cell bodies of the sensory neurons of the peripheral nervous system. In the chick, the peak of sensory neurogenesis and differentiation in the DRG occurs at embryonic day E4.5, at which DRG are considered immature, containing nascent neuroblasts and mitotically active progenitor cells. By E8.5, DRG are considered mature, containing post-mitotic differentiated neurons, resident glia, and Schwann cells. To identify molecules regulating sensory neurogenesis and differentiation, we directly compared immature E4.5 DRG to mature E8.5 DRG through a subtraction/differential screening methodology. Preliminary screenings of candidate cDNAs confirmed that they are differentially expressed. We have extensively characterized the in vivo function of one candidate, neural epidermal growth factor-like like 2 (NELL2), a novel secreted glycoprotein, whose role was previously unknown. In ovo microinjection/electroporation of eukaryotic expression vectors driving ectopic NELL2 expression demonstrate that NELL2 promotes neural differentiation autocrinely and stimulates neighboring cells to proliferate, a novel function for this class of molecule and the first demonstrated in vivo function for NELL2. Furthermore, two receptor tyrosine kinases c-Eyk and c-Rek, members of the same RTK family expressed during sensory neurogenesis, have been investigated as to their roles during DRG development. Preliminary analysis indicates that in particular, Rek loss-of-function exhibits a unique phenotype whereby transfected cells cluster in the dorsal pole of the DRG, as if they were inhibited from differentiating. Further experimentation on these receptors is in progress. Finally we are currently developing new technologies to allow general loss-of-function approaches, including dominant negative, DNAenzyme, and RNAi mediated strategies for protein knockdown in ovo.

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GLOSSARY AND ABBREVIATIONS

14-3-3ζ	Zeta isoform of the 14-3-3 family of cytosolic integrators of signal transduction pathways identified in the subtraction screen.
26S ATPase cS4	One subunit of the regulatory cap complex of the 26S proteosome identified in the subtraction screen.
9e10	Monoclonal antibody specific to the Myc antigen.
BDNF	Brain Derived Neurotrophic Factor
Ben	Monoclonal antibody specific to a cell surface antigen, possibly carbohydrate, expressed by neurons and used as a general neural marker.
bHLH	basic Helix-Loop-Helix family of transcription factors
BMP	Bone Morphogenetic Protein, a family of secreted morphogen that patterns the dorsal neural tube
BrdU	Bromodeoxyuridine, a nucleotide analog that is incorporated into replicating DNA, and then used as a marker of S phase proliferating cells with an anti-BrdU antibody.
CMV IE	Cytomegalovirus immediate early enhancer
CNS	Central Nervous System
COS7	Transformed monkey kidney cell line commonly used for eukaryotic protein expression.
Delta/Serrate/Jagged	Transmembrane ligands for the transmembrane receptor Notch, involved in lateral inhibition (neural versus glial decisions).
DMEM	Dulbecco's modified Eagle's medium, supplemented with fetal bovine/calf serum
DRG	Dorsal root ganglion, spinal ganglia that contains the cell bodies of all trunk peripheral sensory neurons.
DsRedI	Novel red fluorescent protein cloned from the Discostoma coral by virtue of its homology to GFP, used as a reporter.

E4.5	Embryonic day 4.5, that is 4.5 days of incubation of a fertilized chicken egg
ECM	Extra-Cellular Matrix
EGF	Epidermal Growth Factor
ER	Endoplasmic reticulum
EST	Expressed sequence tag
ETS	Family of transcription factors containing ETS-domains
Eyk	East Lansing Tyrosine Kinase, a RTK that belongs to the Axl/Tyro/Mer family of RTKs that is expressed in DRG.
G3PDH	Glyceraldehyde 3-phosphodehydrogenase
GFP	Enhanced green fluorescent protein from the bioluminescent jellyfish Aequoria Victoria, used as a reporter.
GST	Glutathione S-transferase, an affinity fusion tag for protein purification.
H3	Histone 3 marker for M phase mitotically active cells.
HcRedI	Far-red fluorescent protein isolated from the reef coral *Heteractis crispa.*
HSPG	Cell surface heparin sulfate proteoglycans
IP	Immunoprecipitation
IRES2:eGFP	Internal ribosomal entry site 2:enhanced GFP expression cassette that allows independent translation of a coding DNA sequence cloned upstream, and eGFP, used as a reporter.
Isl1	Islet1, a member of the LIM homeodomain transcription factor Family
Mash1	Mammalian acheate-scute homolog-1, a member of the bHLH family of proneural transcription factors, vertebrate homologue of Drosophila acheate-scute

Math1	Mammalian atonal homolog-1, a member of the bHLH family of proneural transcription factors, vertebrate homologue of Drosophila atonal
MNR2	A homeodomain transcription factor that is sufficient to direct somatic motor neuron differentiation.
Mrg	MAS-related genes, G protein-coupled receptors (GPCRs) for noxious stimuli expressed by nociceptors.
MyoD	A bHLH transcription factor that is the terminal muscle determination factor.
NELL1	Neural epidermal growth factor-like like 1, a novel secreted glycoprotein named for its EGF-like domains and expression in neural tissues, which is ~50% homologous to NELL2.
NELL2	Neural epidermal growth factor-like like 2, a novel secreted glycoprotein named for its EGF-like domains and expression in neural tissues, which is ~50% homologous to NELL1.
NeuroD	A bHLH transcription factor that is the terminal neuronal determination factor.
NGF	Nerve Growth Factor
Ngn 1/2	Neurogenin 1 and 2, members of the bHLH family of proneural transcription factors, vertebrate homologs of Drosophila atonal.
NKL	Neural Kruppel Like, transcription factor involved in promoting neuronal differentiation.
Notch	Transmembrane receptor for the transmembrane ligands Delta/Serrate/Jagged, involved in lateral inhibition (neural versus glial decisions).
NT-3	Neurotrophin 3
NT-4/5	Neurotrophin 4/5
N-TSP1	N-terminus Trombospondin 1

OLIG1/2	bHLH transcription factors involved in motor neurons and then oligodendrocyte development.
$p75^{NTR}$	Low affinity neurotrophin receptor
Pax 3/7	Paired box transcription factors
PKC	Protein Kinase C
pMIW	Parent eukaryotic expression that contained the original NELL2 coding sequence, created by removing NELL2 from pMIWC3 (C3 is the clone name for NELL2) with HindIII, and religating; contains the dual Rous Sarcoma Virus Long Terminal Repeat (RSV LTR) and chicken beta-actin promoters.
PNS	Peripheral nervous system
Rek	Retina expressed tyrosine kinase, a RTK that belongs to the Axl/Tyro/Mer family of RTKs that is expressed in DRG.
RNAi	RNA interference, a method for knocking down protein levels
RTK	Receptor Tyrosine Kinases
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
ShH	Sonic hedgehog, a secreted morphogen that patterns the ventral neural tube.
SSH-PCR	Suppressive Subtractive Hybridization PCR
St	Normal stages of chick development described by Hamburger and Hamilton (HH)
TrkA	High affinity RTK for NGF
TrkB	High affinity RTK for BDNF
TrkC	High affinity RTK for NT3
Tuj1	Beta-III tubulin, a neural specific tubulin used as a neural marker.
vWC	von Willebrand Factor C domain

ABSTRACT

Vertebrate neurogenesis occurs through the restricted expression of subsets of genes to discrete cell types that organize into the central and peripheral nervous systems during specific developmental periods. A major goal of developmental biology is to understand the molecular and cellular mechanisms underlying this complex process. An experimentally rich system in which to investigate this question is the dorsal root ganglion (DRG), which derives from a population of migrating neural crest cells that aggregate laterally to the neural tube, and contain the cell bodies of the sensory neurons of the peripheral nervous system. In the chick, the peak of sensory neurogenesis and differentiation in the DRG occurs at embryonic day E4.5, at which DRG are considered immature, containing nascent neuroblasts and mitotically active progenitor cells. By E8.5, DRG are considered mature, containing post-mitotic differentiated neurons, resident glia, and Schwann cells. To identify molecules regulating sensory neurogenesis and differentiation, we directly compared immature E4.5 DRG to mature E8.5 DRG through a subtraction/differential screening methodology. Preliminary screenings of candidate cDNAs confirmed that they are differentially expressed. We have extensively characterized the *in vivo* function of one candidate, neural epidermal growth factor-like like 2 (NELL2), a novel secreted glycoprotein, whose role was previously unknown. *In ovo* microinjection/electroporation of eukaryotic expression vectors driving ectopic NELL2 expression demonstrate that NELL2 promotes neural differentiation autocrinely and stimulates neighboring cells to proliferate, a novel function for this class of molecule and the first demonstrated *in vivo* function for NELL2. Furthermore, two receptor tyrosine kinases c-Eyk and c-Rek, members of the same RTK family expressed during sensory neurogenesis, have been investigated as to their roles during DRG development. Preliminary analysis indicates that in particular, Rek loss-of-function exhibits a unique phenotype whereby transfected cells cluster in the dorsal pole of the DRG, as if they were inhibited from differentiating. Further experimentation on these receptors is in progress. Finally we are currently developing new technologies to allow general loss-of-function approaches, including dominant negative, DNAenzyme, and RNAi mediated strategies for protein knockdown *in ovo*.

1. INTRODUCTION

Vertebrate neurogenesis occurs through the restricted expression of subsets of genes to discrete cell types that organize into the central and peripheral nervous systems during specific developmental periods. This process occurs through the intricate interplay of extrinsic and intrinsic signals that generate a diverse set of neurons and glia from common progenitor populations (reviewed in Morrison, 2001a; Sommer, 2001). A major goal of developmental biology is to understand the molecular and cellular mechanisms underlying this complex process, and much of what has been learned to date began as studies of simpler systems. Early investigations have predominately focused on identifying intrinsic components of this regulatory system, by identifying transcription factors conserved across phylogeny. In *Drosophila* the two proneural basic Helix-Loop-Helix transcription factors *achaete-scute* and *atonal* have been shown to be responsible for the production of the peripheral nervous system external sensory organs and chordotonal organs respectively, while their vertebrate counterparts *mammalian acheate-scute homolog-1 (Mash1)* and the *neurogenins (Ngn)*, which are related to *atonal*, are necessary and sufficient for the differentiation of peripheral autonomic neurons of the sympathetic ganglia and sensory neurons of the dorsal root ganglia, both derivatives of the neural crest (reviewed in Anderson, 1999). Proneural gene expression is transient in progenitors and upregulates downstream family members, such as NeuroD, which drive these cells to differentiate into neurons, analogous to the pro-muscle transcription factor family whereby upstream factors induce MyoD, the terminal muscle determination factor (Perez et al., 1999; Brunet and Ghysen, 1999).

In the developing CNS, the spinal cord has served as an excellent model system because of its well-defined topology, where mitotically active progenitor cells are located in a single-cell layer in the ventricular zone, surrounded by an intermediate and mantle zone where post-mitotic cells migrate laterally to differentiate (Leber et al., 1990). Mash1, ngn1/2, and other members of the bHLH family of proneural transcription factors such as mammalian atonal homologs Math1 are also expressed in the central nervous system, in largely non-overlapping patterns of gene expression confined to the ventricular zone. Discrete patterns of proneural gene expression within the spinal cord suggest that not only are proneural genes sufficient to permit progenitors to differentiate into neurons, but that they might also play a role in specifying neural identity (Sommer et al., 1996). Indeed different members of the proneural bHLH gene family can contribute to specification of neural identity, for example Math1 specifies dorsal interneuron subpopulations (Johnson, 2002). However genetically swapping bHLH coding sequences has revealed that some of these factors are more generally permissive rather than instructive for the formation of neurons, especially the NGNs (Parras et al., 2002). Furthermore proneural genes induce neural differentiation by simultaneously inducing downstream neural factors and sequestering glial-promoting complexes specifically (reviewed in Morrison, 2001b).

Nevertheless different types of progenitor cells must exist such that some give rise to neurons, some give rise to glia, and some give rise to both neurons and glia (Leber et al., 1990), and regulated so that neurogenesis precedes gliogenesis. Accordingly these proneural progenitor cells differentiate into specific types of neurons as the result of the overlapping pattern of homeodomain transcription factor code, which is generated by graded concentrations of secreted ventralizing sonic hedgehog ShH and dorsalizing BMP

signaling, the major identified extrinsic signals in the spinal cord (Briscoe et al., 2000; Briscoe and Ericson, 2001). These homeodomain transcription factors form precise domains within the ventricular zone through the overlapping expression of pairs of cross-inhibitory class I and class II partners. Within a given domain, the co-expression of particular bHLH proneural genes and homeodomain genes results in the differentiation of discrete types of neurons, which are born at precise times of development (reviewed in Lee and Pfaff, 2001).

This is an elegant story for the production of neurons in the CNS, however it must be more complex than this, as at some point the glial derivatives must also be generated from these progenitor domains. Examination of the progenitor cells in these domains reveals that virtually all cells in a given domain express a given homeodomain transcription factor and a proneural transcription factor. The proneural genes are reciprocally regulated through the classic lateral inhibition pathway mediated by the transmembrane signaling receptor Notch and its transmembrane bound ligands Delta/Serrate/Jagged (reviewed by Gaiano and Fishell, 2002). The process of lateral inhibition allows for the selection of non-equivalent cells from a field of otherwise homogenous cells types. In the presumptive nervous system, the expression of Delta in a given cell results in the upregulation of proneural genes and Delta itself in that cell, which in turn signals through Notch on neighboring cells to downregulate proneural and Delta gene expression in surrounding cells, keeping them in an undifferentiated state. Accordingly Notch is expressed throughout the spinal cord ventricular zone, and its ligands are expressed in largely non-overlapping patterns in this zone, suggesting that different Notch ligands and different subsets of proneural genes are regulated in a dorso-ventral pattern as well, although what

mediates this pattern is not currently known (Ma et al., 1997). Analysis of the expression of these genes within a given domain demonstrates that the majority of progenitor cells must co-express Notch and one of ligands at the same time, as each gene appears to be expressed by the majority of cells in that domain. Co-expression of Notch in inner ear precursors and the differential expression of Delta and Serrate results in the production of hair cells and support cells, demonstrating that progenitor cells can widely express Notch and then utilize its different ligands to sort out cell fates (Eddison et al., 2000). Therefore within a given domain in the spinal cord, progenitor cells differentiate into specific classes of neurons based on the overlapping patterns of Notch and its ligands, and the proneural and homeodomain transcription factors.

A major question then is how does this neural differentiation program relate to glial specific differentiation programs? Recently it was demonstrated that they could be directly coupled. The motor neuron specific progenitor domain in the ventral spinal cord is defined by the coexpression of NGN2 and the transcription factors OLIG1/2 from the homeodomain code, originally named for their expression pattern that overlaps with a domain that generates the glial derivatives oligodendrocytes at later stages (reviewed in Kessaris et al., 2001; Zhou and Anderson, 2002). Early in development, this combination results in the birth of all motor neurons, defined by expression of motor neuron determination transcription factor MNR2, which migrate laterally and ventrally to a region outside of the ventricular zone, giving rise to the nascent motor pools. Later, after motor neurons have been generated, NGN2 is downregulated in this domain through an unknown mechanism, which then allows OLIG1/2+ progenitor cells to switch from generating motor neurons to give rise to oligodendrocytes (Zhou and Anderson, 2002). This suggests that an

as-of-yet unidentified extrinsic/intrinsic signal tells the progenitor domains to stop generating neurons and start generating glial derivatives at precise times during development, and that some progenitors remain in these domains to generate these later arising cell types.

Extrinsic and intrinsic mechanisms are also important for the development of the peripheral sensory nervous system that is generated from a subpopulation of neural crest cells (reviewed in Morrison, 2001a; Sommer, 2001). An experimentally rich system in which to investigate this question as it pertains to vertebrate neurogenesis is the dorsal root ganglion (DRG), and is the system that we have utilized in our studies. Neural crest cells are induced to form at the boundaries between the neural plate and epidermal ectoderm during gastrulation. After neural tube closure, the neural crest migrates out of the dorsal neural tube along well-defined routes whereby subpopulations of crest generate different peripheral derivatives (Lallier and Bronner-Fraser, 1988). Mature DRG contain approximately twenty types of post-mitotic neurons that differ in their sensory modalities, morphologies, and biochemistry, in addition to support glial cells (Scott, 1992). Immature ganglia contain a less well-defined population of cells consisting primarily of nascent, undifferentiated neurons and, importantly, mitotically active progenitor cells that can generate neurons and/or glial cells.

In the nascent DRG, the first-born post-mitotic differentiating neurons aggregate and cluster in the core of the ganglia, ensheathed by mitotically active progenitor cells (Lallier and Bronner-Fraser, 1988). Classic cell-lineage studies of neural crest demonstrate that indeed, these are a multipotent stem cell-like population *in vivo*, with certain crest able to generate all subpopulations of peripheral cell types (Fraser and Bronner-Fraser, 1991;

Frank and Sanes, 1991). However it is becoming clear that subpopulations of crest exist with a more restricted lineage *in vivo*, such that some give rise to sensory rather than sympathetic ganglia, and within a given ganglion, some generate different types of neurons and/or glia (reviewed in Anderson, 1999, 2000; Morrison, 2001a, 2001b; Sommer, 2001). For example high concentrations of the extrinsic signaling molecule BMP promotes neural crest to differentiate into sympathetic specific cells, and is expressed by the dorsal aorta endothelial cells *in vivo*, adjacent to the sympathetic ganglia anlagen. This in turn upregulates the proneural gene Mash1, which is necessary and sufficient to drive crest to differentiate into sympathetic neurons when ectopically expressed. Similarly low BMP concentrations promote neural crest to differentiate into sensory rather than sympathetic cells, and result in the upregulation of the proneural genes NGN1/2, which are necessary and sufficient to promote neural crest to differentiate into sensory ganglia specific cells; DRG fail to form in mice with null mutations in both of these genes (Ma et al., 1999). Single mutations in either NGN2 or NGN1 demonstrate that each proneural gene is expressed by a subpopulation of neural crest sensory precursors, that NGN2 is responsible for the early wave of neurogenesis producing TrkC and TrkB neurons, that NGN1 is responsible for a later wave of neurogenesis producing most if not all TrkA neurons, and that these separate subpopulations can compensate for the loss of each other (Ma et al., 1999). This demonstrates a genetic correlate for a heterogeneic sensory neural crest subpopulation, and accordingly overexpression of either NGN in neural crest cells biases them towards a sensory fate (Perez et al., 1999).

However the precise roles of the NGNs in the development of the peripheral sensory nervous system remains unclear, as revealed by recent fate-mapping studies

demonstrate in which cells transiently expressing Ngn2 are permanently labeled. These studies demonstrate that NGN2+ sensory specific neural crest cell subpopulation gives rise to both neurons and glia with no bias, and no apparent bias in sensory neural subpopulations either (Zirlinger et al., 2002). This seems to contradict these previous studies that have shown that NGN2 gives rise to TrkB and TrkC sensory neural subpopulations early and NGN1 gives rise to the TrkA subpopulation later (Ma et al., 1999), and therefore further experiments are needed to elucidate this apparent discrepancy. It was also shown that while NGN2 could permit Mash1-depependent sympathetic precursors to initiate sympathetic ganglion formation, it could not support their proliferation and further development (Parras et al., 2002), and it normally is not expressed in these cells *in vivo* (Perez et al., 1999; Parras et al., 2002; Zirlinger et al., 2002).

Indeed much of what we have learned about the development of DRG has come from the classic studies of the later effects of neurotrophins and the roles of their cognate Trk receptors during target regulated programmed cell death (Hamburger, at al., 1981; reviewed by Farinas et al., 2002). Mature avian DRG that have undergone retrograde neurotrophin transport and target regulated programmed cell death contain a well-defined organization where the first born large diameter neurons (TrkB+ and TrkC+) are located ventro-laterally, and the later-arising small diameter (TrkA+) neurons are located dorso-medially. Interestingly in the course of investigating the role of NGF in the survival of this dorso-medial population of neurons, Hamburger and colleagues described the location in which these neurons originate as being in the medial dorsal. Small diameter neurons accumulated in this region and displaced the larger neurons towards the ventro-lateral region of the ganglion (Hamburger et al., 1981). This population of small diameter

neurons is TrkA+, and genetically arises from the NGN1 precursor pool which undergoes its peak period of neurogenesis and differentiation later at ~E6 (Ma et al., 1999). Immunoreactivity for TrkA expression at E4.5 demonstrates that indeed a population of nascent neurons in the dorsal region are TrkA+, however these nascent neurons actually co-express other neurotrophin receptors, in particular TrkC, and exhibit very dynamic neurotrophin receptor switching during development (Rifken et al., 2000). NGN1 gene expression is confined to the dorsal pole and the ensheathing cell layer progenitor domains (Perez et al., 1999; Nelson and Lefcort, unpublished). Furthermore the downstream proneural gene NeuroD is also restricted to the progenitor domains at these later stages, and not in nascent neuroblasts in the core of the ganglia that widely express pan-neural markers (Nelson and Lefcort, unpublished). Other studies investigating neuron glial decisions in the ensheathing cell layer propose that, as neurons (and perhaps glia) are born they migrate into the core to differentiate (Wakamatsu et al., 2000). This raises the question then, as where exactly do neurons and glia originate in the DRG, and where do they end up? In other words, do neurons and glia intermingle with other nascent neurons in the core, or do they instead pack around the core in layers or groups in neurons? Although not the specific topic of this dissertation, this intriguing question is currently understudy (Nelson and Lefcort, unpublished).

While much is known about the development of peripheral sensory neurons, the set of identified molecules and their functions cannot fully account for the generation of such diversity nor explain this process. We have utilized this system to identify new molecules involved in regulating sensory neurogenesis and differentiation, by designing a subtraction screen to identify genes specifically expressed in immature E4.5 DRG, at a stage that

represents the peak period of neurogenesis and differentiation. The candidate molecules identified in our preliminary analyses have not been previously identified in this tissue. Extensive characterization of one candidate, neural epidermal growth factor-like like 2 (NELL2) has demonstrated a unique expression pattern and intriguing *in vivo* function, the first demonstrated for this family of secreted glycoproteins. We have also studied the *in vivo* function of two receptor tyrosine kinases during DRG development, c-Eyk and c-Rek, members of the same RTK family of receptors. Finally we have sought to develop techniques for *in ovo* knockdowns, some of which should prove to be very useful and expand the range of the chick model system in experimental embryology.

2. IDENTIFICATION OF GENES REGULATING SENSORY NEUROGENESIS AND DIFFERENTIATION

Abstract

The dorsal root ganglia (DRG) derive from a population of migrating neural crest cells that coalesce laterally to the neural tube. As the DRG matures, discrete cell types emerge from a pool of differentiating progenitor cells. To identify genes that regulate sensory genesis and differentiation, we have screened the transcriptomes of immature and mature DRG. Several differentially expressed genes were identified in these analyses that belong to important regulatory gene families. One molecule we identified is a secreted glycoprotein, neural epidermal growth factor-like like 2 (NELL2), which we found to be exclusively expressed in the immature DRG, in addition to exhibiting a strikingly dynamic expression pattern in the developing spinal cord and hindbrain. Confirmation of the differential expression of NELL2 and other genes identified in our screen demonstrate the usefulness of this approach for isolating key regulatory genes dynamically involved in the genesis and differentiation of discrete cell types and tissues (Nelson et al., 2002a).

Introduction

A central question in biology is how a multicellular organism, consisting of an extremely diverse population of cell types, can arise from a single cell. The restricted expression of subsets of genes to a particular tissue during specific developmental stages ultimately results in the differentiation of discrete cell types. The goal is to identify key

genes that regulate such fundamental events as the genesis and differentiation of these discrete cell types. An experimentally rich system in which to investigate this question as it pertains to vertebrate neurogenesis is the dorsal root ganglion (DRG), which derives from a population of migrating neural crest cells that aggregate laterally to the neural tube. Mature DRG contain approximately twenty types of post-mitotic neurons that differ in their sensory modalities, morphologies, and biochemistry, in addition to support glial cells (Scott, 1992). Immature ganglia contain a less well-defined population of cells consisting primarily of nascent, undifferentiated neurons and importantly, mitotically active progenitor cells that can generate neurons and/or glial cells. Molecular differences between immature and mature ganglia will be reflected in their respective transcriptomes and proteomes. Identification of these molecular differences is key to understanding how such diversity arises, as well as to elucidate how developmental programs can go awry to result in sensory neural pathologies.

Once DRG have formed, sensory neurons mature and innervate discrete central and peripheral targets, followed by an extensive period of target-regulated programmed cell death. However, the intervening cellular and molecular events *prior* to the period of target-mediated cell death, yet *subsequent* to neural crest migration, remain incompletely characterized. An elucidation of these events is required because it is during this time period when all of neurogenesis, and the majority of differentiation of discrete classes of sensory neurons occur. Certainly the neurotrophins (NGF, BDNF, NT-3, NT-4/5) and their receptors (TrkA, TrkB, TrkC and $p75^{NTR}$) play critical roles during sensory neurogenesis (Lindsay, 1996), while the basic-helix-loop-helix transcription factors Neurogenin 1 and Neurogenin 2 are required for the formation of DRG, as mice with null

mutations in both of these genes do not form DRG (Ma et al., 1999). In addition, restricted expression of members of the ETS family of transcription factors can influence the functional identity of subsets of sensory neurons (Lin et al., 1998; Arber et al., 2000), and Notch and Delta-1 interactions can modulate neural/glia fate determination in the developing DRG (Wakamatsu et al., 2000). Though these molecules play important roles during DRG development, they alone cannot fully account for the developmental processes underlying the diversity of sensory neural fate decisions. Thus the goal of this study was to identify genes involved in sensory neurogenesis and differentiation in the nascent DRG by searching for genes with a highly restricted developmental expression pattern.

A powerful experimental strategy to identify genes that regulate specific developmental events is to characterize the transcriptome of a tissue at a particular developmental stage. The identification of differentially expressed genes during development begins by either comparing the ensemble of transcripts or the ensemble of proteins from a particular immature tissue to those from the mature tissue. Three previous transcriptome analyses have been performed on the DRG. Akopian and Woods, (1995) employed a subtraction scheme whereby a mixture of non-neural tissue derived cDNA and neural tissue derived cDNA (driver) was subtracted from postnatal rat DRG cDNA (tester). This resulted in the identification of transcripts specifically or highly expressed in the peripheral sensory nervous system. However because the tester was derived from postnatal DRG, the molecular cues that exclusively govern sensory neurogenesis would not be identified because the DRG had already completed neurogenesis by the time the subtraction was performed. A second experimental

approach utilized RNA fingerprinting to identify genes specific to the NT-3 or NGF dependent subpopulations of mature sensory neurons (Friedel, et al. 1997). This resulted in the identification of transcripts differentially expressed in one of these two subpopulations. However, transcriptomes were derived from mature DRG excised several days after the period of neurogenesis, and then cultured for 4 days, thus reducing the likelihood of identifying genes whose function is to regulate sensory neurogenesis, as well as potentially altering their transcriptome by isolating them from their normal embryonic environment. A third recent experiment utilized suppressive subtractive hybridization PCR (SSH-PCR) to compare neonatal DRG from wild type to Ngn1 -/- mice, in which most TrkA+ neurons, including the nociceptive subclass, fail to generate (Dong, et al. 2001). This resulted in the identification of transcripts known to be expressed in TrkA+ neurons, including a new family of G protein-coupled receptors (GPCRs) termed *MAS-related genes (mrgs)* that are specifically expressed in subpopulations of nociceptors. Although this approach exemplifies the utility of combining SSH-PCR and knockout strategies, it still does not address the problem of identifying molecules that regulate DRG neuron genesis and differentiation.

To this end, our strategy was designed to identify genes that function specifically during sensory neurogenesis and differentiation. We utilized SSH-PCR to directly compare the *in vivo* transcriptome of immature E4.5 chick DRG to the in vivo transcriptome of mature E8.5 DRG (Diatchenko et al., 1996). The genes identified in our screens were not found in the previous DRG transcriptome analyses. Our study has revealed several intriguing molecules whose function during DRG development we are now in the process of determining.

Results

DRG derive from migrating neural crest cells that coalesce laterally to the neural tube beginning at ~ Embryonic Day 2.75-3 in the chick (E2.75-3; Lallier and Bronner-Fraser, 1988). Neurogenesis in the nascent DRG then ensues, peaking at ~ E4.5-5, which is followed by target innervation and programmed cell death of post-mitotic neurons between ~ E5-E12, peaking between E7-E9 (Carr and Simpson, 1978). At E4.5, DRG are immature with ca 30% of the cells being mitotically active progenitor cells (Lefcort, unpublished observations), and the majority of the remaining cells being nascent, post-mitotic neurons (Rifkin et al., 2000: Wakamatsu et al., 2000). Gliogenesis becomes prevalent after E6 (Carr and Simpson, 1978). In order to identify genes that regulate sensory neurogenesis and differentiation we isolated DRG from embryos at E4.5, and compared their cDNA to cDNA derived from mature DRG (E8.5) consisting of post-mitotic, well-differentiated neurons and glia (Nelson, B.R., Anderson, L.W., Kasemeir, J., Lefcort, F., 2002, manuscript in preparation).

Full-length Phage E4.5 chick DRG cDNA Library

We began our investigations by creating a full-length phage E4.5 chick DRG cDNA library (Stratagene) for use as a tool with which to obtain full-length clones identified from our screenings. Results from initial screenings with alpha-tubulin and TrkC probes determined that this library does indeed contain large inserts and a sufficiently high titer and complexity, with appropriate frequencies for a highly abundant transcript (alpha-tubulin and moderately abundant transcripts (TrkC and Brn3A; Table 1). Furthermore, additional cDNAs have been identified through PCR based screening for

members of the proto-cadherin, Brn3A transcription factor, and anaplastic lymphoma kinase (ALK) family of RTKs.

Table 1. Full-length phage E4.5 Chick DRG cDNA Library			
Titer = 10^8 plaque forming units/ml			
cDNA	Method	% Frequency	Insert size
Alpha-tubulin	probe	2.8% 140/5000 clones	1.3-2.3kb
TrkC	probe	0.18% 37/20,000 clones	1-4kb
Brn3A	probe	0.137% 11/8000 clones	
Proto-cadherins	PCR		
Alk	PCR		

Subtraction analysis

The *in vivo* transcriptomes of E4.5 and E8.5 DRG were directly compared with a suppressive subtractive hybridization PCR (SSH-PCR) / differential screening methodology (Lukyanov et al., 1995; Munroe et al., 1995; Diatchenko et al., 1996; von Stein et al., 1997). We have initially screened approximately 600 clones from our E4.5 subtracted library with probes derived from total, unsubtracted cDNAs from both ages, and the subtracted cDNAs from both ages. Figure 1 shows the typical results of our differential screening. The top long arrow points to an amplicon from a clone (identified as neural epidermal growth-factor like like 2, NELL2) having different signal intensities

with these probes, indicating that SSH-PCR has significantly enriched NELL2 at E4.5 and that NELL2 is much less expressed at E8.5. The bottom row is a series of control amplicons, and the lower right arrow points out the subtraction of the glyceraldehyde 3-

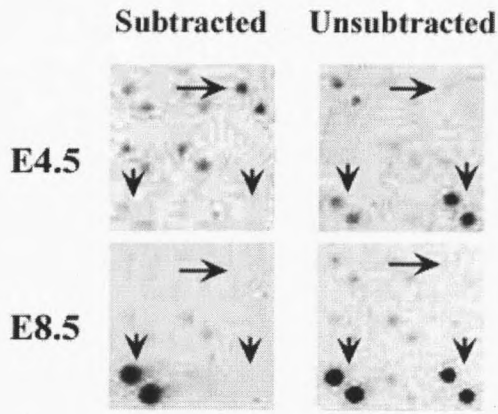

Figure 1. Differential screening. Films from quadruplicate arrays of amplified cDNAs from the E4.5 DRG subtraction library hybridized with E4.5 subtracted and unsubtracted, and E8.5 subtracted and unsubtracted P^{32} labeled probes. Top right long arrow indicates a clone identified as NELL2. Bottom right short arrow is the subtraction control G3PDH. Bottom left short arrow indicates a clone identified as Neuroserpin.

phosphodehydrogenase (G3PDH) amplicon in both subtracted probe sets, while strong signals are seen in both unsubtracted probe sets, indicative of the success of SSH-PCR. The amplicon in the lower left corner (left arrow) is a random clone picked from the E8.5 subtracted library as a reverse control, showing that it was significantly enriched by the reverse experiment and, interestingly, was later identified as Neuroserpin, a gene known to be involved in and expressed in later stages of neural differentiation (Osterwalder et al., 1996; Krueger et al., 1997).

Analysis of the SSH-PCR experiment indicates that 1) G3PDH, a highly abundant house-keeping gene present in both E4.5 and E8.5 transcriptomes was efficiently subtracted out, 2) clones from the E4.5 subtraction library show differential signal

Table 2. Differentially expressed cDNAs identified by SSH-PCR		
cDNA	Embryonic Day	# of clones
Neural Epidermal Growth Factor-Like Like 2 (NELL2)	E4.5	41
14-3-3ζ / YWHAZ / *Leonardo*	E4.5	13
26S ATPase complex Subunit 4	E4.5	13
Novel	E4.5	1
Neuroserpin	E8.5	1

intensities indicating that enrichment occurred for a subset of E4.5 specific genes in the forward experiment, and 3) clones from the E8.5 subtraction library show differential signal intensities as well, indicating that the reverse experiment was successful in enriching for E8.5 specific genes. This demonstrates that our transcriptome analysis effectively enriched differentially expressed transcripts and subtracted common, housekeeping transcripts. Table 2 lists the candidate differentially expressed genes chosen on the basis of their specifically enriched signal in the E4.5 subtraction library, ~70 candidates were sequenced. We are continuing to screen this library by probing it with a mixture of probes made by PCR-DIG labeling of previously identified clone fragments, which has already yielded a new novel candidate cDNA. Furthermore we examine the other subtraction library that is enriched for E8.5 specific cDNAs to identify candidates with potential roles in mature ganglia. Finally we will screen a complementary retina subtraction library (T. Reh), enriched for genes expressed in immature retinal progenitor domains rather than mature differentiating retinal domains,